PATENT
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Applicant: Scott, Ian R.
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For: METHOD TO IDENTIFY BIOLOGICALLY ACTIVE AGENTS AND SYNERGISTIC COMBINATIONS

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West Nyack, New York
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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Ian R. Scott, a citizen of the United Kingdom, residing at Lambourne House, Lower Binton, Stratford upon Avon, Warwickshire, UK, do hereby declare that:

1. I hold the degrees of B.A., M.A. and Ph.D. from Cambridge University and am a member in good standing of The Society for Investigative Dermatology.

2. I am the coauthor of some 26 peer reviewed scientific publications dealing with various aspects of skin biology, biochemistry, skin biophysics and dermatology, a named inventor of some 19 granted United States patents and have given numerous presentations to scientific societies including Society for Investigative Dermatology, Gordon Conferences, International Federation of Societies for Cosmetic Chemistry, International Society for Cosmetic Dermatology.

3. I am presently employed at Synergy Biosystems Ltd. as Director.

4. I have read Scott, U.S. Patent Application S.N. 10/807,811, filed March 24, 2004, of which I am a named Inventor. I have also read the Office Communication mailed November 17, 2006.

5. In this declaration I discuss the differences between my method and prior art methods especially that disclosed by Stockwell et al (US 2002/0019011), illustrate these differences by way of an example, and conclude with a discussion of the advantages of my method.

6. Discovering synergies within a large library of chemicals is daunting because of the very large number of possible combinations. For example, a library of 1000 chemicals can be combined to form approximately: 500,000 binary combinations; 170 million ternary combinations; 42 billion quaternary combinations; 8.3 trillion quintuple combinations and so on for even higher order combinations.

7. The literature reports two basic ways to discover synergistic combinations.

Method (i): Test all possible combinations. This is taught, for example, by Stockwell (para 0100). Because of the large number of experiments involved this is only practical for small libraries combined in low order combinations (binary, tertiary, quaternary etc) – for practical purposes binary combinations only.

Method (ii): Only test combinations of chemicals which by themselves exhibit activity. This is the standard method of seeking synergy and is taught, for example, by Stockwell (para 0017, 0103). This approach can be extended to higher order combinations by combining binary combinations that show synergy (Stockwell para 0103). In this method the number of tests required to discover synergistic combinations is reduced but the method suffers a fundamental problem in that it does not discover combinations of chemicals where one of the chemicals fails to show activity on its own, or, for higher order combinations, fails to show synergy in lower order combinations.

8. In contrast, my method can interrogate large libraries for higher order synergistic combinations while keeping the number of tests required to a practical level. This method does not eliminate chemicals that may ultimately be critical components of the most effective combination merely because they show little or no efficacy on their own or in lower order combinations.

9. The differences between my method and the prior art can best be demonstrated in two stages – firstly by contrasting what is actually experimentally done in my method compared to the prior art and secondly by illustrating these differences by way of an example.

10. My experimental approach compared with the prior art. There are two key differences between my method and methods of the prior art (Item 7 above). These are the way combinatorial test cycles are constructed; and the use of single and multi-component scaling protocols between cycles. These aspects are discussed below.

Construction of combinatorial test cycles

In the first cycle of my method each chemical in the library is tested separately in the assay and some number are found to have activity.

In the second testing cycle of my method, some fraction of the set of active compounds (the number dictated by practical limits on the number of assays but if necessary only 1 might be used) is then tested in combination with substantially all the remaining members of the library. This step is in stark contrast to either of the prior art methods describe under items 7(i) or 7(ii) above and is counterintuitive as it may ignore some effective chemicals while retesting chemicals that have already failed to show activity.

In the third cycle of my method, some fraction of the effective synergistic binary combinations (again dictated by practical limits on the number of assays, but if necessary as low as 1) is selected and tested again in combination with substantially the entire library of chemicals. This is again in sharp contrast with either of the prior art methods describe under items 7(i) or 7(ii) above. It is again counter intuitive as it involves ignoring a potentially vast number of effective synergistic binary combinations (some discovered in this step and some not discovered because of the combinations of chemicals that were never tested from step 1) while continuing to retest chemicals which showed no effect either alone in the first cycle or in binary combinations in the second cycle.

The process can be continued with 4^{th}, 5^{th} and higher cycles (corresponding to 4 component, 5 component and still higher order mixtures) until either no further increase in activity is found or the activity level reaches a useful point where the combination of chemicals discovered up to that stage is taken for further development. Again this repetition is quite different from the repetition described for example by Stockwell (para 0013-0016) wherein he

merely repeats an assay with different subsets of the large number of possible combinations being investigated. In my method, in contrast to Stockwell, the repetition potentially increases the order (i.e., binary, ternary, quaternary etc) of the combinations by 1, each time a cycle is added and *continually involves retesting chemicals that have repeatedly failed to show activity.*

Use of Single and Multiple Component Scaling Protocol between cycles

The second key methodological difference between my method and prior art methods lies in the use of Single and Multiple Component Scaling Protocols (MCSP) at the end of each cycle of the method. I will focus on the MCSP. The use of the MCSP is fundamentally different to the concentration adjustments, i.e., the *dilution matrix* described, for example, in Borisy et al (US 2002/0165261).

Borisy merely sets up an array of compound concentrations to ensure that the individual chemicals are present within the array at levels where their activity is accurately measurable both when the chemicals are tested individually and also when tested in combinations.

In contrast, as stated on page 11, lines 11-26 of my specification, the MCSP protocol is a dose response study of the identified mixture whereby the concentration of each component of the mixture is independently varied. These protocols are carried out between combinatorial cycles. Such multi-component dose response studies and their computerized analysis are well known in the field of high-throughput testing (e.g., genome project).

The MCSP has two distinct functions essential to maximizing both the effectiveness and efficiency of my approach.

Firstly, the MCSP adjusts the concentration of the chemical or chemicals to be used in subsequent combinations so as to exhibit a relatively low activity in

the assay. With this adjustment only a single test concentration in the next cycle round is sufficient to determine if synergy is being shown for any single combination of chemicals. This arises because the MCSP ensures that the single test combines a combination of chemicals being taken forward at a combined concentration ensuring low activity, with a single chemical from the library that also shows no or low activity. This approach obviates the need for *compound dilution arrays* as taught by Borisy. This greatly reduces the experimental load by dramatically cutting back on the number of experiments per cycle.

Secondly, the MCSP is used to eliminate chemicals from the combinations being taken forward to subsequent cycles as described in my specification on page 12 lines 21 onwards. This approach both streamlines testing and ensures that the mixture with greatest synergy is identified. Without the MCSP methodology the number of components in the synergistic mixture would always increase by 1 chemical for every new cycle of testing. However, this ignores the very real possibility that as new compounds are added to the synergistic mixture in further testing cycles, chemicals already present in the mixture may now cease to contribute to synergism.

The consequence of this can best be illustrated through the following simple example. Suppose the 4^{th} cycle of testing involving the combination of chemicals A, B, and C with the remaining library identifies that the addition of component D yields a quaternary mixture having still higher synergy. Without using the MCSP, the combination of ABCD would be taken forward to cycle 5 (5-component testing). However, suppose that applying the MCSP to the ABCD mixture (four dimension dose response testing) reveals that there are combinations of just B, C and D which are not outperformed by any of the combinations containing A. Chemical A is therefore not effectively contributing to synergism when D is present and A can eliminated from the mixture.

Since in most applications, large numbers of cycle repetitions would be expected to be used (see below) this elimination of chemicals that no longer contribute to the efficacy of the combination is an important aspect of the invention.

11. Example illustrating differences from prior art testing. The power of my method lies in the fact that at every cycle substantially the entire library is retested with the combinations selected to be taken forward. Thus, if in fact the "ultimate" most effective combination contains chemical X, then after sufficient cycles chemical X will inevitably be selected. This is because, so long as X is not in the combination, there will always be a further combination (not necessarily containing X) that has an increased activity over the combination taken forward and that will continue to be so until X is selected.

A hypothetical example helps to clarify this point and quantitatively point out the differences between my method and prior art approaches to synergy testing. The example is based on one among the possible mechanisms whereby real synergy is thought to be achieved in complex biological systems: namely, where two (2) pathways control a particular biochemical process but one of these pathways is redundant (a backup pathway), i.e.,

> Pathway 1 is a conventional "rate limiting pathway" so that inhibition of that pathway results in immediate alteration of the systems behavior.
>
> Pathway 2 is a "redundant backup" which only comes into play when Pathway 1 is inhibited.

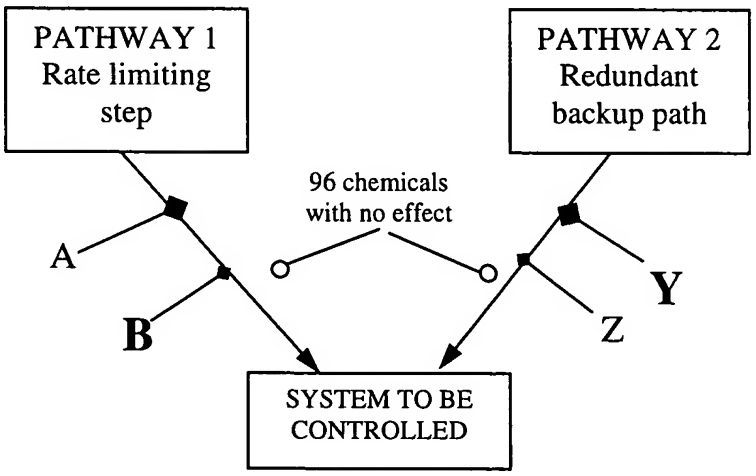
Inhibition of Pathway 2 has no effect on the system unless Pathway 1 is already inhibited but if both Pathway 1 and Pathway 2 are inhibited, a greater effect on the system can be achieved than through even complete inhibition of Pathway 1. This is true synergy.

ASSUMED FACTS

Out of a library of 100 chemicals only chemicals **A** and **B** inhibit Pathway 1 while **Y** and **Z** inhibit Pathway 2.

Only chemicals A and B are active by themselves with chemical A being more effective than chemical B. Both Y and Z form synergistic mixtures with either A or B with chemical Y producing a greater level of synergy with either A or B. However, the most effective synergistic combination in the library is a mixture of B and Y (**BY**) even though A on its own is more effective than B.

This system is illustrated below.



Most effective combination **BY**

TESTING ACCORDING TO MY METHOD

Given the above facts scenario (which are of course not known at the beginning of testing) the interrogation of this library by my method would proceed according to the following table below.

STEP	STAGE	NUMBER OF TESTS	POSITIVE RESULTS (active chemicals and mixtures)	TAKEN FORWARD TO NEXT STEP
1	Cycle 1 – **all chemicals are tested alone**	100	A, **B**	A, **B**
2	Single component scaling protocol	10	A,**B**	A
3	Cycle 2 – **A + Library**	100	AY, AZ , AB	AY, AZ, AB
4	Multiple component scaling protocol	75	AY, AZ	AY
5	Cycle 3 – **AY + Library**	100	AYB, AYZ	AYB, AYZ
6	Multiple component scaling protocol	250	**YB**, AY	**YB**
7	Cycle 4 – **YB + Library**	100	**YB**A, **YB**Z	**YB**A, **YB**Z
8	Multiple component scaling protocol	250	**YB**	End – **YB** selected
	Total Number of Experiments	**985**		

Cycle 1 identifies A and B since they are the only effective chemicals in the library. Dose response studies at 5 concentrations are then carried out using the single component scaling protocol – step 2. This reveals that A is more effective than B. Chemical A is taken forward for binary testing with the remaining members of the library.

Cycle 2 identifies AY,AZ and AB as having activity greater than A alone. A 2-dimensional dose response study is carried out using the multiple component scaling protocol (step 4). This shows that in AB, B is redundant in that there are concentrations of A at which B adds nothing to the activity of the mixture. AY and

AZ show real synergy and AY is selected to be taken forward to the next cycle as it has the higher overall activity.

Cycle 3 identifies AYB and AYZ as having greater activity than AY alone. These mixtures are taken forward to the multiple component scaling protocol which shows that in AYB, A is redundant (in that there are combinations of YB having equal activity to any combination of AYB) while in AYZ, Z is similarly redundant. AY was the input to the previous cycle so YB is taken forward to the next cycle.

Cycle 4 identifies YBA and YBZ as having higher activity than YB. These mixtures are taken forward to the multiple component scaling protocol (step 8) which shows that in YBA, A is redundant while in YBZ, Z is redundant.

YB is therefore selected as a candidate for in-vivo testing. The method has avoided missing the contribution of Y and Z which had no activity in the first cycle.

The unexpected occurrence of a "crossover" synergy in this example (A being better than B when tested alone but B being better than A in the synergistic mixture) meant that the initial choice of A as the chemical to take forward to the second round of testing simply because it was the most effective on its own, was incorrect. However, this error merely caused the process to extend by an additional cycle before identifying the optimum synergistic mixture. Any simple error that resulted in an inferior chemical or combination being taken forward, or the arbitrary choice of one rather than another equally effective mixtures being taken forward would similarly be corrected by the extension of the process with additional cycles. The process is thus experimentally robust, which is of critical importance in high throughput experimentation.

COMPARISON WITH OTHER METHODS

In the above illustration, a library of 100 chemicals was interrogated by my method. The most effective binary combination was identified AND it was confirmed that no ternary combinations were more effective. The screening process required 985 experiments.

The two prior art methods (i) and (ii) discussed in Item 7 applied to the above example would give very different outcomes either in terms of actually identifying the most synergistic combination or in terms of the experimental load required to identify it.

Method (i) 1 – test all possible combinations

To test all possible binary combinations at one concentration of each compound would require (100 x 99)/2 = 4950 assays. Furthermore, without the use of a step analogous to the Single Component Scaling Protocol as in my method, the testing of each component of the library at multiple concentrations (e.g, "compound dilution matrix" as used by Borisy et al - page 15, paragraph 0145) would further increase the number of tests by a factor of 25 to 123,750 assuming that each compound were tested at 5 concentrations.

In terms of output, method (i) would identify all the synergistic binary combinations including the best combination BY. To confirm that this combination was more effective than any ternary combination would require an additional 100 x 99 x 98/6 = 161,700 assays or 4,042,500 assays if multiple concentrations were tested.

Thus, even with a relatively small library of 100 compounds, method (i) of Stockwell et al would require over 4,000 times more experimentation to reach the

same conclusions as was reached by applying my method. The differences become enormous as the library size increases.

Method (ii) Only test combinations of chemicals which by themselves exhibit activity

The method would fail to discover the synergistic combination which involve Y and Z since these chemicals were eliminated after the first stage of testing because they exhibit no activity by themselves. Thus, the method fails to identify the most effective synergistic mixture BY.

12. Speaking as an expert in skin science, my method is distinct from prior art methods in terms of the specific practical steps taken and represents a valuable advance in the art of discovering synergies among biologically active chemicals. As items 10 and 11 above illustrate my method requires fewer assays to discover synergies within a library of chemicals than the prior art methods and is able to discover the most effective synergistic combination of chemicals from a library irrespective of how many different chemicals from the library must be combined into a single combination to achieve the maximum efficacy. Since my method requires no mechanistic understanding of the system being controlled it is quite general in utility.

13. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this patent application or any patent issuing thereon.

Dated: .....8th February 2007

Ian R. Scott